

A Simple Derivation of the Luminescence Anisotropy Decay from Randomly Distributed Cylinders Rotating About a Single Axis

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Abstract A simple derivation is given of the expression describing the anisotropy decay of luminescence for a solution of molecules that can only undergo rotational diffusion about a single cylindrical axis. The usual derivations of the anisotropy decay for this cylindrical model have simply taken limiting cases of the equations resulting from the general treatment of the anisotropy decay of a completely anisotropic rotator or the rotation of an ellipsoid. The arguments presented here can be understood without the mathematical sophistication required to follow the general derivations for the rotational diffusion of a completely anisotropic rotator or ellipsoids. The underlying physical mechanisms leading to a multiple exponential decay of the fluorescence anisotropy signal from a single axis rotating cylinder are clearly shown by following this derivation. The resulting expression for the anisotropy decay is not new. However, the derivation is easily understood, and this article is meant as an introduction to the more advanced treatments of anisotropy decay by rotational diffusion. After presenting the derivation of the rotating cylinder, the corresponding steps of these general treatments and this simple model are indicated. The model is of special interest for describing the anisotropy decay resulting from rotations of proteins within membranes.

Keywords Fluorescence anisotropy decay · Rotation diffusion · Cylinder · Derivation · Physical mechanisms · Observed components

Introduction

This is an introductory account, involving a simple model of rotational diffusion, to better understand the more complex models describing the time course of an anisotropy signal measured from a fluorescent sample that has been excited by a light pulse. Fluorescence, phosphorescence and absorption of spectroscopic reporter molecules are the most common spectroscopic parameters for which the anisotropy is measured. In biological experiments it is common to attach a small spectroscopic reporter molecule to larger molecules in order to acquire information about the rotational freedom of these complexes either free in solution or in conjunction with other macromolecular structures. Such measurements have been applied to the study of macromolecules that are embedded in biological membranes, free rotations of molecules in solution, and bending, torsional twisting, and internal motions of molecular groups relative to the structure of a larger “host” structure. The functional form of the time dependence, the quantitative decay times and the magnitude of the anisotropy changes indicate the size and shape of the rotating species, the viscosity or other dissipative effects of the surroundings, and physical restrictions, which may retard the rotational freedom of the rotator.

However, the information from such measurements is limited by the practical difficulties in analyzing the anisotropy curves, and by the difficulty in choosing an appropriate model for interpreting the data. The solution for the decay of the luminescence anisotropy of a freely rotating rigid hydrodynamically anisotropic body has been described in general [1–4]; the signal consists of five exponentially decaying terms. The derivations in these publications parallel previous work [5–8], and a more recent account considering the theory for extended applications can be consulted [9]. If restrictions hinder the rotational freedom of even very

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symmetrical molecules, more complicated functions are required to describe the time dependence of the anisotropy [10]. The difficulties of an analysis in terms of these general models are usually circumvented by considering the anisotropy decay of simpler models, such as the free rotation of a sphere (one exponential) or an ellipsoid (three exponentials), or restricting the rotations to one axis only (two exponentials). It is the last model, the rotation about one molecular axis, which is the topic of this work.

The simple model of a cylinder rotating only about its symmetry axis has been invoked to interpret the results of experiments involving the rotations of elongated proteins, DNA [11, 12] and molecules in membranes [13]. The mathematical description of the luminescence anisotropy decay of a rigid cylinder diffusing about only one cylindrical axis has not been derived from first principles. It is easily obtained by setting all the rotational diffusion constants, except one, to zero in the general description of a freely rotating anisotropic rigid body (or rotating ellipsoid). This correctly predicts two exponential anisotropy decays [11], and the exponential time constants differ by exactly a factor of four. However, even though the resulting mathematical expression is correct, the physical process underlying the two decay phenomena is not evident from this type of derivation. One might think that only a single anisotropy decay constant should correspond to a single axis of rotation, because the cylinder undergoes a simple rotational diffusion movement around only a single axis. This seems reasonable by analogy to translational diffusion along a single Cartesian axis [14–17].

These general theoretical derivations of the time dependent fluorescence anisotropy for a completely anisotropic freely rotating body [2–4] are straightforward, but mathematically complex. For those unfamiliar with Wigner rotation matrices, spherical harmonic functions and Legendre polynomials, the details of these calculations, which embody the physics of the problem, remain obscure. Even the general solution of the rotational diffusion equation for a freely rotating sphere, which has only a single exponential anisotropy decay, involves an expansion in spherical harmonics [18] or associated Legendre polynomials [16, 19]; the excitation and emission processes photoselect only a single relaxing component from an infinite number of individual components. Why a cylinder rotating about its cylinder axis requires two time constants to describe the Brownian anisotropic decay, even though only one diffusive motion is involved, is not obvious. Even if one studies in detail the derivation for an anisotropic rotator, it is non-trivial to understand the correspondence between the separate rotational axes and the observed number of components of the anisotropy decay.

Theoretical treatments of the fluorescence anisotropy decay for an arbitrary, anisotropic free rigid body begin with the rotational diffusion equations that were first solved by Favro [1]. Favro's landmark study does not treat the observation

of rotations by spectroscopic means, but presents a general solution to the diffusion equation. The treatments of [2, 3] use the equations and ideas of Favro as starting points for their discussions. The sophisticated presentation by Favro is extremely difficult, if not practically impossible, to follow without advanced mathematical prerequisites. The later extensions of Favro's work to derive expressions for fluorescence anisotropy decay, although more amenable to a larger audience provided that one accepts the starting premise of Favro's work, are still very challenging to understand. The result is that most experimental practitioners simply use the elegantly presented equations by [2–4], and amend them to fit their presumed models. This is certainly a valid procedure; however, as discussed above, understanding the relation between the components of the anisotropy decay and the symmetry of the rotating object is not straightforward, even in the case of a simple cylinder undergoing rotational diffusion around only its cylinder axis. For instance, the question arises whether the separate exponentially decaying components can be assumed to arise from some physical motion, or is it a result of the method of carrying out the experiment.

A description of a simple case, which is not based upon these more difficult general treatments, would be helpful. Derivations of a freely rotating sphere, without referring to the results for anisotropic rotators, have been presented [14, 20, 21]; indeed, the derivations for spherical rotators were published long before the general case for anisotropic rotators was presented. In his second paper on the theory of Brownian movement [14, 22] Einstein discussed the rotational diffusion of a sphere. He gave the equation for the averaged squared angular deviation of the polar angle (the angle between the laboratory z -axis and the selected vector fixed within the sphere) as a linear function of time, $\langle(\Delta\phi)^2\rangle_{av}/t = kT/(4\pi\eta r^3) = D_r$, where D_r is the rotational diffusion constant for a sphere. This was later derived explicitly by Debye [20, 23], in relation to the analysis of dielectric dispersion experiments. This simple model for spheres undergoing free rotational diffusion has been widely applied to interpret experiments of fluorescence anisotropy decay. The derivation involves the expression of the angular movement in terms of limiting small increments. The derivation for the anisotropy decay of rotating ellipsoids is much more complex [5, 19, 24].

The topic of this report is a simple derivation of the anisotropy decay for a rotating cylinder diffusing only around its cylindrical axis. The derivation is easy to follow (involving only algebra and some trigonometry), and it also shows clearly the coupling between the physical model of molecular rotation and the experimental method of observation. Analogies can then be drawn with the more general, complicated models of rotational diffusion and anisotropy decay.

The goal of this paper is more pedagogical. The resulting expression for the time dependent anisotropy decay

for a cylinder rotationally diffusing only about its cylinder axis, Eq. (10a), is not new. The results are identical to previous publications. When deriving the expression for the anisotropy decay of this model, only knowledge of Fourier Series and trigonometric manipulations is required. Therefore, many of the important and interesting correlations between the anisotropy measurement and the theoretical derivations, which are also present in the general theoretical treatments of the more complex cases, can be appreciated by a larger audience.

Parameterization of the problem

Figures 1 and 2 define the parameters used to describe the rotation of a molecule with arbitrarily directed absorption and emission dipoles about the molecular cylindrical axis (\hat{n}). The polar angle, φ , describes the deviation of the molecular axis \hat{n} from the \hat{z} axis of the laboratory coordinates frame. θ is the angle between the \hat{x} axis and the projection of the \hat{n} axis

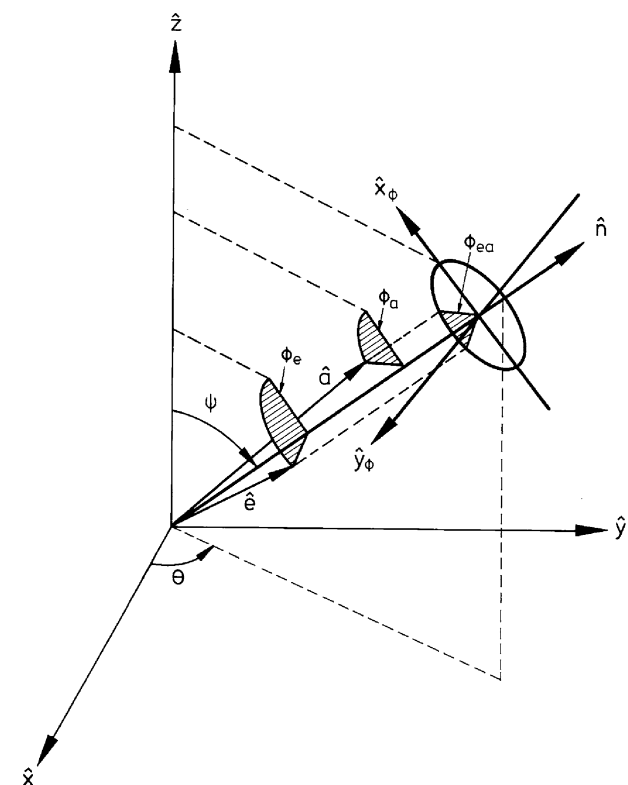


Fig. 1 Right-handed vector coordinate system defining the orientation of the cylindrical rotation axis (A), the absorption dipole (\hat{a}) and the emission dipole (\hat{e}). θ_a and θ_e are the angular deviations of the absorption dipole and the emission dipole, respectively, from the \hat{n} axis. The electric field of the exciting light is orientated parallel to the \hat{z} axis. The \hat{x}_ϕ vector indicates the x molecular axis of the cylinder. This axis is defined for every molecule such that it is perpendicular to the \hat{n} vector and is orientated toward the laboratory z axis (see the text). Details of the figure are given in the text

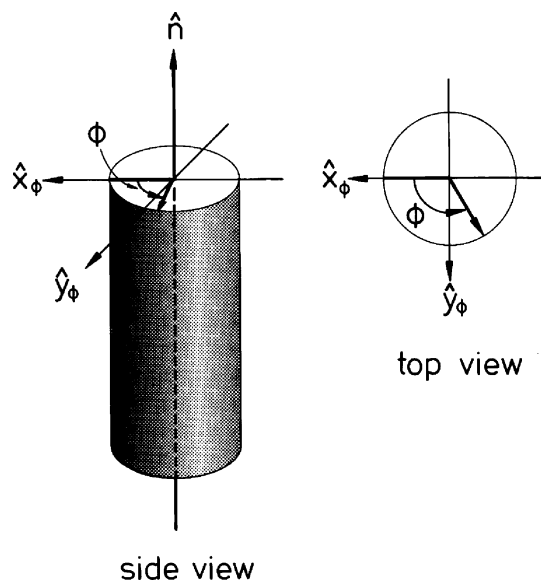


Fig. 2 Coordinate of the cylindrical system. The \hat{z} vector is identical to the \hat{n} vector. The \hat{x}_ϕ vector is always oriented toward the laboratory \hat{z} axis as discussed in the legend for Fig. 1 and the text. The angle Φ depicts the azimuthal angle of an arbitrary vector of the molecular system within this cylindrical coordinate system

onto the $\hat{x}\hat{y}$ plane. Thus φ and θ are the normal polar angles representing the orientation of the \hat{n} axis relative to the laboratory $\hat{x}\hat{y}\hat{z}$ coordinates. The values of φ and θ are random within the ensemble of molecules, but they are assumed to remain constant for each molecule for the time of observation (restricted motion, see Fig. 2); however, each molecule is free to rotate about the molecular \hat{n} axis. Φ is the azimuthal angle describing the angular orientation of any vector in the molecular coordinate frame about the \hat{n} axis. The angle, Φ , for each molecule, is defined within the plane perpendicular to the \hat{n} vector for that molecule (see Figs. 1 and 2), and Φ increases in the direction of the vector cross product, $\hat{n} \times \hat{z}$. The $\Phi = 0$ position for any molecular vector, \hat{a} , is defined by maximizing the dot product, $\hat{a} \cdot \hat{z}$, where \hat{a} is a molecular vector (such as the absorption \hat{a} and emission \hat{e} transition dipoles), which can rotate about \hat{n} . The vector, \hat{x}_ϕ of Figs. 1, 2, and 4, defines the direction where $\Phi = 0$ within the plane perpendicular to \hat{n} . \hat{y}_ϕ , which is defined in Fig. 4, is consistent with a right-handed coordinate system with the \hat{n} vector as the z-axis. The molecules all have different values of \hat{n} , x_ϕ , and \hat{y}_ϕ , each with corresponding values of the angles φ , θ and Φ . These angles are closely related to the Euler angles describing the rotation within Cartesian coordinate systems [25, 26]. The $\Phi = 0$ position has been defined differently than for the corresponding Euler angle, because we define $\Phi = 0$ for each φ and θ separately. We use the generic Φ to indicate the angular position of a selected position within a cylindrical molecule, referring to its rotational position. Specifically, we define the angle for the absorption and emission dipoles relative to the molecular \hat{n} axis about which the

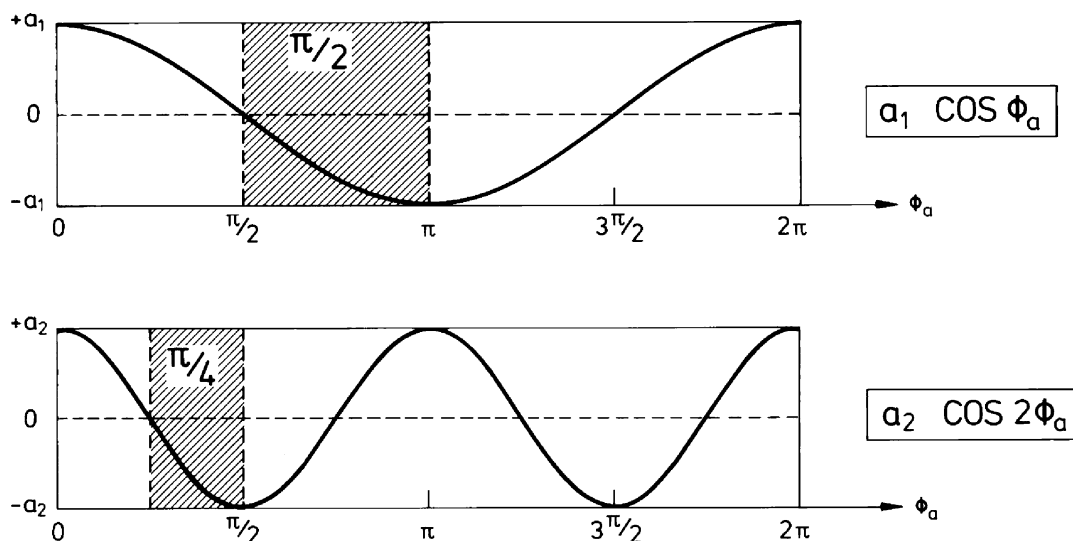


Fig. 3 The two Fourier components of the angular distribution of the excited population of molecules. The shaded areas indicate the angular displacements of the two components separating the extremes and the minima of the distributions

molecule rotates as $\Phi = \phi_a$ for absorption, and as $\Phi = \phi_e$ for emission.

Diffusion equation and general solution

The rotational diffusion equation for a cylinder rotating about the cylindrical axis is easily obtained from the general isotropic rotational diffusion equation with angular coordinates [5, 15, 18–20] by disregarding the terms involving changes in the orientation of the cylindrical axis. The result is the differential equation

$$D_\Phi \frac{\partial^2}{\partial \Phi^2} P(\Phi, t) = \frac{\partial}{\partial t} P(\Phi, t) \tag{1}$$

where $P(\Phi, t)$ is the probability that the cylinder has an angle Φ at time t provided that the distribution is defined by $P(\Phi, 0)$ at $t=0$. D_Φ is the rotational diffusion constant about the molecular axis \hat{n} . This model is also applicable to a symmetrical ellipsoid rotating around only one axis; one has only to use the correct rotational diffusion constant. Since the rotational diffusion constant is $D_\phi = kT/f_r$ [22], where k is the Boltzmann’s constant, T is the temperature in Kelvin, and f_r is the rotational diffusion constant about the rotation axis. A convenient list of f_r for different geometries can be found in the book by Berg [17] and in Memming [5]. Note that equation 1 is simply the one dimensional diffusion equation, in terms of the angular coordinate Φ . The dependence of $P(\Phi, t)$ upon φ and θ need not be considered to analyze the diffusion phenomenon since we assume that φ and θ remain constant. Equation (1) holds for every distribution over Φ , for each particular φ and θ . $P(\Phi, 0)$ does depend upon φ and

θ ; however, we will eventually integrate the total molecular ensemble distribution over all values for these angles.

A particular real solution to Eq. (1) is

$$(a_k \cos(k\Phi) + b_k \sin(k\Phi))e^{-k^2 D t},$$

where a_k and b_k are constants. $P(\Phi, t) = P(\Phi + 2\pi, t)$, so k must be an integer. Since Eq. (1) is linear, a more general

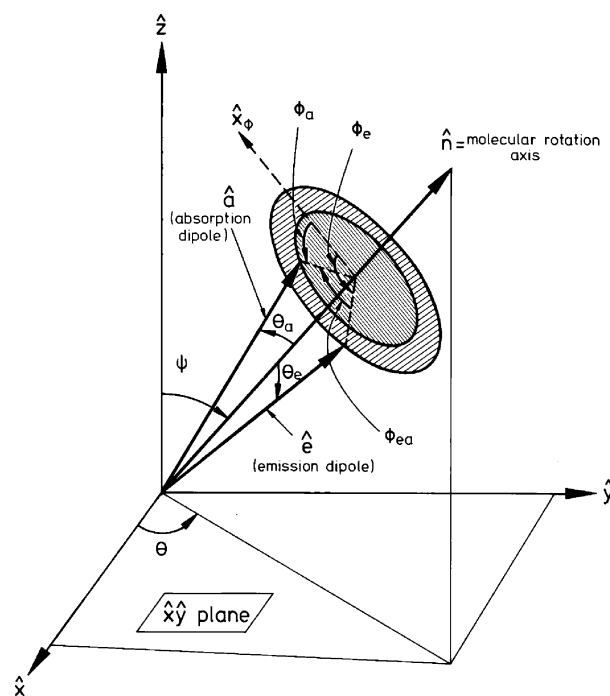


Fig. 4 The coordinate system of Fig. 1 clarifying the orientations of the emission dipole, \hat{e} , and the absorption dipole, \hat{a} , and the azimuthal angle between them, Φ_{ea}

solution is a sum of all possible particular solutions given above, which is a Fourier series expansion.

$$P(\Phi, t) = a_0 + \sum_{k=1}^{\infty} (a_k \cos(k\Phi)) + (b_k \sin(k\Phi)) e^{-k^2Dt} \quad (2)$$

The initial distribution at $t = 0$ is a related Fourier series and can be used to solve for the a_k and b_k according to

$$P(\Phi, 0) = a_0 + \sum_{k=1}^{\infty} (a_k \cos(k\Phi)) + (b_k \sin(k\Phi)), \quad (3)$$

where, according to the theory of Fourier expansions

$$a_0 = \left(\frac{1}{2\pi}\right) \int_{-\pi}^{\pi} P(\Phi, 0) d\Phi \quad (3a)$$

$$a_k = \left(\frac{1}{\pi}\right) \int_{-\pi}^{\pi} P(\Phi, 0) \cos(k\Phi) d\Phi \quad (3b)$$

$$b_k = \left(\frac{1}{\pi}\right) \int_{-\pi}^{\pi} P(\Phi, 0) \sin(k\Phi) d\Phi \quad (3c)$$

Therefore, in order to find the general solution to the rotational diffusion equation, Eq. (1), we only need to know the initial distribution $P(\Phi, 0)$. Equations (3a)–(c) are used to calculate the Fourier coefficients, and Eq. (2) represents the time dependence of the angular probability distribution in terms of a series of exponentials.

General observations about the general solution

We have not yet considered anisotropy experiments, which will define $P(\Phi, 0)$ by photoselection (the above equations refer only to the molecular rotation, not to the observation of the rotation), but several essential features of the problem are already apparent.

(a) The number of exponential time constants representing the distribution of rotations of any chosen vector within the molecular coordinate system, $P(\Phi, 0)$, is equal to the number of different orders of the Fourier coefficients left after considering the photo-selection of excitation and emission detection; that is, for every k in Eq. (2), there is a corresponding exponential time decay. If $P(\Phi, 0) = a_n \cos(n\Phi) + a_m \cos(m\Phi)$ then the time dependence is $P(\Phi, t) = a_n \cos(n\Phi)e^{-n^2Dt} + a_m \cos(m\Phi)e^{-m^2Dt}$. The photoselection process defines the initial distribution, $P(\Phi, 0)$ for $\Phi = \phi_a$, and ϕ_e (see section c below), and determines the maximum number

of absorption or emission components decaying exponentially in time. This is the maximum number, since without knowledge of the absorption and emission photoselection processes, the general expression of Eq. (3) extends over a greater number of Fourier components. $P(\Phi, 0)$ and $P(\Phi, t)$ do not consider the observation of the anisotropy (that is, passing the fluorescence through an emission polarizer), which will usually reduce the number of the originally excited components considerably. $P(\Phi, 0)$ and $P(\Phi, t)$ only represent the probability distribution of the angular distribution of the excited absorption or emission dipoles, and their time dependent changes. Some of these (most) exponentially decaying components of $P(\Phi, t)$ cannot be observed when measuring the anisotropy decay, as we will see.

- (b) The inverse of the time constants are proportional to k^2 . This is an expected result of Einstein/Schmoluchowski diffusion processes defined by Eq. (1), which involve the second derivative of the coordinates and the first derivative of the time (see section c below) [14, 15, 17, 20, 27].
- (c) There is a simple heuristic argument describing the form of Eq. (2). Assume that $P(\Phi, 0) = a_0 + a_1 \cos(\Phi) + a_2 \cos(2\Phi)$, which will be shown to be the case for the absorption transition dipoles that are selected by photoselection with linearly polarized light. Figure 3 represents such an initial Φ distribution in graphic form. According to Eq. (2) each sinusoidal distribution in Fig. 3 will decay exponentially with time; the $k = 2$ term decays four times faster than the $k = 1$ term. The angular distance separating the maximums (or minimums) of each curve from the zero values (dotted lines in Fig. 3) is $\frac{\pi}{2}$ for $\cos\Phi$ and $\frac{\pi}{4}$ for $\cos2\Phi$. That is, the angular displacement required to relax to the infinite time distribution (dotted lines in Fig. 3) for the $\cos\Phi$ distribution is double that of the $\cos2\Phi$ term. From the theory of Brownian motion we know that the average of the squared distance traveled during a random walk in one dimension along the x-axis, if the random walk starts at $x = 0$, is given by $\langle x^2 \rangle = 2Dt$, where the brackets denote averages and D is the one dimensional diffusion constant [14, 22]. That is, the time required to traverse a distance is proportional to the square of the distance. Thus we would expect the time required to relax the $\cos\Phi$ distribution to be four times the time required for the $\cos2\Phi$ distribution. Equation (2) shows that this squared dependence holds for all values of k .
- (d) The shape of the separate components of the distribution (sinusoidal in this case) does not change with time, only the amplitudes decrease. This property also holds for the more general cases of rotation, provided the *allowed rotations are not restricted*. In the present case of a cylinder, all values of Φ (referring to the molecular

axes) about the \hat{n} -axis are equally probable at equilibrium (no photoselection). Thus, if the time dependence of the angular distribution is described by a series of exponential terms, the amplitudes of these exponential decays are defined by the photoselection process (which will define the initial distribution of ϕ_a and ϕ_e), and the amplitudes are functions only of these angles (see Fig. 1). However, for the general treatment of anisotropic rotators, some of the amplitudes are also functions of the diffusion constants [2–4].

- (e) These amplitudes are represented in terms of the appropriate angular functions (eigenfunctions of the diffusion operator) for the particular variables and symmetry under consideration. In the present case these functions are simple cosine and sine functions of Φ , but for general spherical isotropic rotational diffusion the functions are the surface spherical harmonics (or rather the corresponding Legendre polynomials, see below), and for anisotropic bodies we need the transformation functions defined by the Wigner rotation matrices. As for the special case above, the angular boundary conditions for the general case result in integer variables, similar to the integer k of Eq. (2), which enter in the exponential terms in combination with the diffusion constants. The normal modes of the decay process for the general case [2–4] do not vary simply as the square of integers times a diffusion constant since the diffusion process can no longer be thought of as independent diffusion relaxations about an axis. One must now consider the angular dependence of the set of functions defined by the Wigner rotation matrices, or the surface spherical harmonics [25, 26, 28], which define the initial distribution.

Initial photoselected distribution

The coefficients, a_k and b_k , of Eq. (2) are defined from the initial distribution, $P(\Phi, 0)$ by Eq. (3). The general case will be treated, where the absorption and emission transition dipoles can be at any particular angles relative to the molecular \hat{n} -axis and to each other. Refer to Figs. 1 and 4 for the angular definitions. The initial distribution of the absorption or the emission dipoles can be derived easily by simple geometrical arguments. The absorption dipoles (with unit vector \hat{a}) are excited by light that is linearly polarized along the \hat{z} laboratory axis, and the probability of absorption for any molecule is proportional to the square of the vector dot product $(\hat{z} \cdot \hat{a})^2$. We express \hat{a} in the coordinate system $(\hat{x}_\Phi, \hat{y}_\Phi, \hat{n})$ in which two of the axes are in a plane perpendicular to the vector \hat{n} (see Fig. 4).

$$\hat{a} = (\hat{x}_\Phi \cdot \hat{a})\hat{x}_\Phi + (\hat{y}_\Phi \cdot \hat{a})\hat{y}_\Phi + (\hat{n} \cdot \hat{a})\hat{n}$$

Using the relationships

$$\begin{aligned}\hat{n} \cdot \hat{a} &= \cos \theta_a & \hat{x}_\Phi \cdot \hat{a} &= \sin \theta_a \cos \Phi_a \\ \hat{n} \cdot \hat{z} &= \cos \varphi & \hat{x}_\Phi \cdot \hat{z} &= \sin \varphi \\ \hat{y}_\Phi \cdot \hat{z} &= 0\end{aligned}$$

we have

$$\hat{z} \cdot \hat{a} = \cos \theta_a \cos \varphi + \sin \theta_a \sin \varphi \cos \Phi_a$$

The distribution of excited absorption dipoles is independent of θ , as we expect because of symmetry about the \hat{z} axis.

Thus we have

$$P(\varphi, \theta_a, \Phi_a) = (\hat{z} \cdot \hat{a})^2 = C_0^a + C_1^a \cos \Phi_a + C_2^a \cos 2\Phi_a \quad (4)$$

where

$$\begin{aligned}C_0^a &= \cos^2 \varphi \cos^2 \theta_a + \left(\frac{1}{2}\right) \sin^2 \varphi \sin^2 \theta_a \\ C_1^a &= 2 \cos \varphi \cos \theta_a \sin \varphi \sin \theta_a \\ C_2^a &= \frac{1}{2} (\sin^2 \varphi \sin^2 \theta_a)\end{aligned}$$

This represents the initial distribution of the ensemble of excited molecules in terms of the orientation of the photoselected absorption dipoles. This would be the correct distribution for a technique, which would observe the change in the population distribution of the absorption dipoles, such as absorption dichroism [19].

For fluorescence or phosphorescence detection, the important initial distribution is given in terms of the photoselected emission dipoles. The initial probability distribution of the emission dipoles of a photoselected samples of excited molecules, $P(\varphi, \theta_e, \phi_e)$ at $t = 0$ is related to $P(\varphi, \theta_a, \phi_a)$ and is obtained by simply replacing θ_a and Φ_a in Eq. (4) by their corresponding expressions in terms of θ_e and ϕ_e .

Since

$$\theta_{ea} = \theta_e - \theta_a \quad \text{and} \quad \phi_{ea} = \phi_e - \phi_a,$$

and

$$\hat{z} \cdot \hat{a} = \cos(\theta_e - \theta_{ea}) \cos \varphi + \sin(\theta_e - \theta_{ea}) \sin \varphi \cos(\phi_e - \phi_{ea}),$$

we have at time zero

$$\begin{aligned}P(\varphi, \theta_e, \phi_e) &= C_0^e + C_1^e \cos \phi_e + C_2^e \cos 2\phi_e \\ &\quad + C_3^e \sin \phi_e + C_4^e \sin 2\phi_e\end{aligned} \quad (5)$$

where

$$\begin{aligned}C_0^e &= \cos^2(\theta_a) \cos^2 \varphi + \left(\frac{1}{2}\right) \sin^2(\theta_a) \sin^2 \varphi \\ C_1^e &= 2 \cos(\theta_a) \sin(\theta_a) \cos \varphi \sin \varphi \cos(\phi_{ea})\end{aligned}$$

$$\begin{aligned}
 &= \left(\frac{1}{2}\right) \sin(2\theta_a) \sin(2\varphi) \cos(\phi_{ea}) \\
 C_2^e &= \frac{1}{2} \sin^2(\theta_a) \sin^2 \varphi \cos(2\phi_{ea}) \\
 &= \frac{1}{8}(1 - \cos 2\theta_a)(1 - \cos 2\varphi) \cos(2\phi_{ea}) \\
 C_3^e &= 2 \cos(\theta_a) \sin(\theta_a) \cos \varphi \sin \varphi \sin(\phi_{ea}) \\
 &= \left(\frac{1}{2}\right) \sin(2\theta_a) \sin(2\varphi) \sin(\phi_{ea}) \\
 C_4^e &= \left(\frac{1}{2}\right) \sin^2(\theta_a) \sin^2 \varphi \sin(2\phi_{ea}) \\
 &= \frac{1}{8}(1 - \cos 2\theta_a)(1 - \cos 2\varphi) \sin(2\phi_{ea})
 \end{aligned}$$

To calculate this distribution, the angle between the absorption and emission transition dipoles must be known (i.e. θ_{ea} and ϕ_{ea}); remember the emission dipoles are initially selected by the polarized excitation light through the corresponding absorption dipoles for each molecule that are selected by the absorption process.

In contrast to the distribution of absorption dipoles we now have an additional dependence upon $\sin\phi_e$ and $\sin 2\phi_e$, since the molecules are selected by the absorption transition dipole, which is not necessarily parallel to the emission dipole. For every ϕ (and θ) the photoselected distribution of dipoles relaxes to the static random distribution with a time dependence given by

$$\begin{aligned}
 P(\phi_e, t) &= C_0^e + (C_1^e \cos \phi_e + C_3^e \sin \phi_e)e^{-Dt} \\
 &+ (C_2^e \cos 2\phi_e + C_4^e \sin 2\phi_e)e^{-4Dt}, \tag{6}
 \end{aligned}$$

according to Eq. (2). Note that the C_i^e and C_i^a are all dependent on the angle φ , but independent of θ (the polar angle of the cylinder axis relative to the z -axis of the laboratory frame), due to our choice of the laboratory z -axis parallel to the direction of polarization of the exciting light. Also, whether we select the relaxation of the absorption or the emission transition dipoles for observation, we find a time dependence involving two exponentials with time constants $1/D_\phi$ and $1/4D_\phi$.

Observing the rotational diffusion by measuring the anisotropy decay

Up to this point we have not yet considered the detection of the rotational diffusion, which is done by observing the luminescence through a linear polarizer along the \hat{y} axis. The axis of the polarizer is oriented parallel to the \hat{z} axis (vertical position) or the \hat{x} axis (horizontal position). The expressions for the observed signals are derived by considering the

projection of the emission dipole (\hat{e}) onto the laboratory \hat{z} (vertical signal) or \hat{x} (horizontal signal) axis. The distribution of the emission dipoles is given for all times by Eq. (6). To obtain an expression for the observed signals this distribution is then multiplied by $(\hat{z} \cdot \hat{e})^2$ or $(\hat{x} \cdot \hat{e})^2$ and integrated over all the available angles of the equilibrium distribution of the molecular \hat{n} axes, since we observe the total ensemble of molecules through the polarizer.

The vertical signal is

$$= \int_0^{2\pi} \int_0^{2\pi} \int_0^\pi P(\varphi, \Phi_e, t) \cdot (\hat{z} \cdot \hat{e})^2 \sin(\varphi) d\varphi d\theta d\phi_e$$

and the horizontal signal is

$$= \int_0^{2\pi} \int_0^{2\pi} \int_0^\pi P(\varphi, \Phi_e, t) \cdot (\hat{x} \cdot \hat{e})^2 \sin(\varphi) d\varphi d\theta d\phi_e.$$

$(\hat{z} \cdot \hat{e})^2$ is given by an equation identical to Eq. (4) except θ_a and ϕ_a are replaced by θ_e and ϕ_e respectively. $(\hat{x} \cdot \hat{e})^2$ is more complicated due to the fact that the projection of \hat{e} onto the \hat{x} axis is not independent of θ . We have, from Fig. 4

$$\begin{aligned}
 \hat{e} &= (\hat{n} \cdot \hat{e})\hat{n} + (\hat{x}_\Phi, \hat{e})\hat{x}_\Phi + (\hat{y}_\Phi, \hat{e})\hat{y}_\Phi \\
 \hat{x} \cdot \hat{n} &= \sin \varphi \cos \theta & \hat{x} \cdot \hat{x}_\Phi &= -\cos \theta \cos \varphi \\
 \hat{n} \cdot \hat{e} &= \cos \theta_e & \hat{y}_\Phi \cdot \hat{e} &= \sin \theta_e \sin \phi_e \\
 \hat{x}_\Phi \cdot \hat{e} &= \sin \theta_e \cos \phi_e & \hat{x} \cdot \hat{y}_\Phi &= \sin \theta
 \end{aligned}$$

We have then,

$$\begin{aligned}
 (\hat{x} \cdot \hat{e}) &= \cos \theta_e \sin \varphi \cos \theta - \sin \theta_e \cos \phi_e \cos \theta \cos \varphi \\
 &+ \sin \theta_e \sin \phi_e \sin \theta = \cos \theta (\cos \theta_e \sin \varphi \\
 &- \sin \theta_e \cos \phi_e \cos \varphi) + \sin \theta_e \sin \phi_e \sin \theta
 \end{aligned}$$

Substituting these expressions into the equations for the vertical and horizontal signals will furnish the expressions we need to calculate the anisotropy decay. The integrations to calculate the observed vertical and horizontal fluorescence polarization components are simplified when we consider the orthogonality of the $\cos(n\phi_e)$ and $\sin(m\phi_e)$ functions. The solutions given by Eqs. (7) and (8) below, are most easily obtained by integrating first over $d\theta$ to simplify the $(\hat{x} \cdot \hat{e})^2$ case, then over $d\phi_e$ to take advantage of the orthogonality of the $\cos(n\phi_e)$ and $\sin(m\phi_e)$ terms, and finally integrating over $d\phi$. Because we allow the angle ϕ_e to have all values, the $\sin(n\phi_e)$ terms of Eq. (6) do not contribute to the amplitude of the signal (the integration over ϕ_e of all terms involving $\cos(n\phi_e)\sin(m\phi_e)$ is zero). This means that there are parts of the photoselected distribution over ϕ_e (i.e. the terms involving $\sin\phi_e$, and $\sin 2\phi_e$), which are relaxing exponentially with time, but which we can not observe with this experimental

method. This characteristic of detection selection is also true for the general anisotropic case. The initial photoselection process selects a distribution that is expressed as a sum over certain of the eigenfunctions of the diffusion operator. In our simple case there are five eigenfunction terms (Eq. (5)). The observation process further selects from the photoselected components only the terms that contribute to the measured anisotropy dynamic signal. Note that even if we limit the range of relaxation of the angle ϕ_e , but have a random initial total distribution of molecules, Eq. (4) still holds, since even a randomly distributed static assembly of molecules is photoselected. However, the observation measurement selects the modes of the *dynamic* relaxation of this initial distribution that we detect as a signal. This would be different than derived in Eq. (5) above if the rotation of each molecule were to be constrained.

The integrations give the following expressions for the projections of the vertical and horizontal signals. These expressions for the projections onto the vertical and horizontal axes are proportional to the signals. We have not considered the spectroscopic, instrumentation or luminescence factors (such as the absorption coefficients or the quantum yields), but all these factors will be the same for the vertical and horizontal signals. If there are instrumentation differences between vertical and horizontal measurements, these are accounted for and corrected experimentally by determining the relative sensitivity factors for the monochromator and detector for the different polarizer positions. These factors will all cancel in the final expression for the anisotropy.

The vertical signal is

$$\begin{aligned} \text{vert. sig.} = & 4\pi^2 \left(\sin^2 \theta_e \left(\left(\frac{4}{15} \right) \sin^2 \theta_a + \left(\frac{2}{15} \right) \cos^2 \theta_a \right) \right. \\ & \left. + \cos^2 \theta_e \left(\left(\frac{2}{15} \right) \sin^2 \theta_a + \left(\frac{6}{15} \right) \cos^2 \theta_a \right) \right) \\ & + \left(\frac{32\pi^2}{15} \right) (\cos \theta_e \sin \theta_e \cos \theta_a \sin \theta_a \cos \phi_{ea}) e^{-Dt} \\ & + \left(\frac{8\pi^2}{15} \right) (\sin^2 \theta_e \sin^2 \theta_e \cos 2\phi_{ea}) e^{-4Dt} \quad (7) \end{aligned}$$

And the horizontal signal is

$$\begin{aligned} \text{hort. sig.} = & 2\pi^2 \left(\sin^2 \theta_e \left(\left(\frac{6}{15} \right) \sin^2 \theta_a + \left(\frac{8}{15} \right) \cos^2 \theta_a \right) \right. \\ & \left. + \cos^2 \theta_e \left(\left(\frac{8}{15} \right) \sin^2 \theta_a + \left(\frac{4}{15} \right) \cos^2 \theta_a \right) \right) \\ & + \left(\frac{16\pi^2}{15} \right) (\cos \theta_e \sin \theta_e \cos \theta_a \sin \theta_a \cos \phi_{ea}) e^{-Dt} \\ & + \left(\frac{4\pi^2}{15} \right) (\sin^2 \theta_e \sin^2 \theta_e \cos 2\phi_{ea}) e^{-4Dt} \quad (8) \end{aligned}$$

The total luminescence signal is independent of the time dependence of the diffusion process, since it is only dependent on the number of luminescent molecules that have been

excited. The total luminescence is given by

$$\begin{aligned} \text{Total luminescence} = & \text{vert. signal} + 2(\text{horiz. signal}) \\ = & \left(\frac{8}{3} \right) \pi^2 \quad (9) \end{aligned}$$

Since all the signals, which we have calculated above, are only proportional to the actual signals, this is the normalization factor. The anisotropy, r , which is the quantity that is usually either measured directly or employed to express the results, is given by

$$r = (\text{vert. signal} - \text{horiz. signal}) / (\text{total signal}) \quad (10)$$

$$\begin{aligned} r = & \left(\frac{1}{10} \right) (3 \cos^2 \theta_a - 1)(3 \cos^2 \theta_e - 1) \\ & + \left(\frac{6}{5} \right) (\cos \theta_e \sin \theta_e \cos \theta_a \sin \theta_a \cos \phi_{ea}) e^{-Dt} \\ & + \left(\frac{3}{10} \right) (\sin^2 \theta_a \sin^2 \theta_e \cos 2\phi_{ea}) e^{-4Dt} \quad (10a) \end{aligned}$$

Equation (10a) is the same as derived by setting all diffusion coefficients except one in the anisotropic rotator, or for the ellipsoid of revolution, to zero. This is conveniently demonstrated in a recent popular book on fluorescence [29]. The equations given in Valeur's book, Eq. (10b), and often in the literature, appear a bit different (Eq. (10b)).

$$\begin{aligned} r = & \left(\frac{1}{10} \right) (3 \cos^2 \theta_a - 1)(3 \cos^2 \theta_e - 1) \\ & + \left(\frac{3}{10} \right) (\sin 2\theta_e \sin 2\theta_a \cos \phi_{ea}) e^{-Dt} \\ & + \left(\frac{3}{10} \right) (\sin^2 \theta_a \sin^2 \theta_e (\cos^2 \phi_{ea} - \sin^2 \phi_{ea})) e^{-4Dt} \quad (10b) \end{aligned}$$

However, using a few trigonometric identities the expressions can be shown to be identical.

Some interesting behavior is predicted by these results. First, the effects of the absorption and emission dipoles are equivalent and symmetrical. Thus if either the emission or the absorption dipole is oriented along the cylinder axis of rotation ($\theta_a = 0$ or $\theta_e = 0$), there will be no anisotropy decay. Both time-dependent terms go to zero no matter what the angle ϕ_{ea} is. In this case one would have no indication of any rotational diffusion—the result would be the same as if $D_\phi = 0$. This happens for two different physical reasons. If the emission dipole is oriented parallel to the cylindrical axis there would be no change in the polarization of the fluorescence emission as the cylinder rotated. However, if the absorption axis is parallel to the cylinder axis, there is a flat

angular distribution of the photoselected emission vectors in the ensemble of molecules; therefore, as the cylinder rotates, although the emission vector of each molecule does change orientation, the ensemble of molecules statistically always retains the uniform distribution of emission orientations. The best way to measure diffusion of the cylindrical axis (lifting the restriction of rotation of the cylinder axis of the molecule through the θ angle) is if $\theta_a = 0$ or $\theta_e = 0$, as then only the rotation of the cylinder axis can be observed; this is shown nicely in the solution for a rotating ellipsoid, where the rotational diffusion of the cylinder axis is allowed (see for instance Valeur [29]). Interestingly, single molecule experiments can detect the rotational relaxation when $\theta_a = 0$, because for each molecule there is a particular orientation of the emission axis for each individual molecule, and its change in orientation by rotational diffusion is observable. If $\theta_e = 0$, rotational diffusion cannot be observed in single molecule experiments; although, different orientations of the individual molecules can be selected through the absorption dipoles. Also, if either of the angles θ_a or θ_e is equal to the “magic angle (54.74 degrees), that is, if the orientation of either the absorption or emission axis is at the magic angle relative to the cylinder axis, the steady state anisotropy ($t = \infty$) is zero. In this case, the components of the anisotropy can either increase or decrease, depending on the relative orientations of the absorption and emission transition moments.

Comparison to the rotation of a sphere

The rotation of a sphere has been commented on above. Although the derivation is more complex than the cylinder rotating around the cylindrical axis, it is relatively simple since it has complete rotational symmetry. Here we will outline a similar procedure up to the event of photoselection in order to show the parallels in the derivation to what we have presented for a rotating cylinder. A nice account of this can be found in the literature [16]. The following derivation will follow closely that of Williams; a similar—more complex—derivation using spherical harmonics, and for ellipsoids, can be found in [19]. Berne and Pecora [18] have given a somewhat different derivation in terms of spherical harmonics. The diffusion equation for the probability that a point on the surface of an isotropic sphere of unit radius is within a solid angle Ω at time t , $f(\Omega, t)d\Omega$, assuming that the direction at $t = 0$ is along the $+z$ -vector, is

$$D_r \nabla^2 f(\Omega, t) = \frac{D_r}{\sin^2 \theta} \times \left[\sin \theta \frac{\partial}{\partial \theta} \sin \theta \frac{\partial}{\partial \theta} (f(\Omega, t)) + \frac{\partial^2}{\partial \phi^2} f(\Omega, t) \right] = \frac{\partial}{\partial t} f(\Omega, t). \tag{11}$$

D_r is the rotational diffusion constant of a sphere, $D_{r, \text{sphere}} = kT/8\pi\eta a^3$, where η is the viscosity of the solvent and a is the radius of the sphere. There is a single diffusion constant (just as for our case of a rotating cylinder about the cylindrical axis) due to of the symmetry of the sphere. Because we are concerned with the photoselection of the absorption transition dipole by the excitation light in a particular direction, the diffusion equation depends only on the polar angle θ and not on the rotational angle ϕ about the z -axis. Then the diffusion equation becomes

$$(1 - \cos^2 \theta) \frac{\partial^2 f(\Omega, t)}{\partial (\cos \theta)^2} - 2 \cos(\theta) \frac{\partial f(\Omega, t)}{\partial \cos(\theta)} = \frac{1}{D_r} \frac{\partial f(\Omega, t)}{\partial t} = (1 - u^2) \frac{\partial^2 f(\Omega, t)}{\partial u^2} - 2u \frac{\partial f(\Omega, t)}{\partial u} \tag{12}$$

where following Williams [16] we substitute $u = \cos \theta$. Similar to our rotating cylinder we can expand $f(\Omega, t)$ in an appropriate set of orthogonal functions; in this case the orthogonal functions are the Legendre polynomials, $P_n(u)$, and we separate the spatial and temporal variables. Each component of the Legendre polynomials will have a corresponding time dependent function—just as we have done for the rotating cylinder.

$$f(\Omega, t) = \sum_{n=0}^{\infty} P_n(u) a_n(t). \tag{13}$$

This solution can be substituted into the differential equation for diffusion, and making use of the expression $(1 - u^2) \frac{\partial^2 P_n(u)}{\partial u^2} - 2u \frac{\partial P_n(u)}{\partial u} = -n(n + 1)P_n(u)$ (where n is an integer; this is the differential equation defining the Legendre polynomials as solutions) [30–32] we have

$$-D_r \sum_{n=0}^{\infty} n(n + 1)P_n(u) a_n(t) = \sum_{n=0}^{\infty} P_n(u) \frac{d}{dt} a_n(t). \tag{14}$$

Equating terms of the same order in the series, leads to the individual equations

$$-D_r n(n + 1)P_n(u) a_n(t) = P_n(u) \frac{d}{dt} a_n(t). \tag{15}$$

The solution to this can be solved by separating the spatial and temporal variables, giving

$$a_n(t) = a_n(0) \exp(-n(n + 1)D_r t). \tag{16}$$

And the solution to the diffusion equation for the probability function $f(\Omega, t)$ is

$$f(\Omega, t) = \sum_{n=0}^{\infty} P_n(\cos \theta) a_n(0) \exp(-n(n + 1)D_r t). \tag{17}$$

We will not follow this derivation for the sphere further, which would lead us to expressions for $a_n(0)$ and to the expression for the anisotropy decay. This is extensively discussed in the literature. But we note that the solution for the diffusion process involves an infinite sum over components of the orthogonal functions appropriate for the symmetry, and the higher components relax faster, just as for our rotating cylinder. It turns out that for fluorescence anisotropy the only component left in the entire sum is for $n=2$. This is heuristically reasonable, because the probability that the transition dipoles will be excited by light polarized along the z -axis, or observed with a polarizer oriented along the z -axis, is proportional to $\cos^2\theta$, and $\cos^2\theta = 1/3(2P_2(\cos\theta) + 1)$. Therefore the sphere will rotate with a rotational correlation time of $\tau_r^{\text{sphere}} = 1/(6D_r)$, as derived originally by Perrin [33]. A nice account of rotation of a sphere and ellipsoids employing incremental rotations, can be found in an article by Weber [34]; this follows the methodology in an earlier article by Soleillet [35].

Recapitulation and comparison to the general case of anisotropic rotators

This section is mainly for those who want to look at the more advanced derivations of the anisotropy decay for completely anisotropic rotators. The theoretical work of [2, 3] (the publication by Belford et al. [4] presents only the results, and does not show their derivation), hereafter referred to as ER and CE respectively, have both used the Green's function approach to solve the diffusion differential equation. Green's function methods are closely related to the method of Fourier employed in here. A lucid discussion of the relation of these two mathematical approaches can be found in [36], pp. 55–62, 71–79 and 177–188. Heuristically, the difference in the two methods is an interchange in the order of integration and summation. The diffusion of a general anisotropic rigid rotor is described by the diffusion equation

$$\frac{d}{dt} f(\Omega, t) = -L f(\Omega, t) \quad (18)$$

where $f(\Omega, t)$ is the distribution function at time t and solid angle coordinates Ω , and L is the corresponding diffusion operator. This equation is analogous to Eq. (1). The general solution of Eq. (18) can be written as a series expansion in terms of the appropriate normalized eigenfunctions, Ψ_n , of L .

$$\sum_n a_n \Psi_n(\Omega) e^{-(\lambda_n t)} = f(\Omega, t) \quad (19)$$

where

$$a_n = \int_{\Omega_0} f(\Omega_0) \Psi_n^*(\Omega_0) d\Omega_0 \quad (20)$$

$\lambda_n = n$ th eigenvalue of L

The subscript “0” refers to the zero time condition. We can write Eq. (19) into the form of a Green's function solution by interchanging the order of the integration and summation to give:

$$f(\Omega, t) = \int_{\Omega_0} f(\Omega_0) G(\Omega_0, \Omega, t) d\Omega_0 \quad (21)$$

where

$$G(\Omega_0, \Omega, t) = \sum_n \Psi_n^*(\Omega_0) \Psi_n(\Omega) e^{-(\lambda_n t)} \quad (22)$$

is the “Greens function” (see [30]). At time $t=0$ the Green's function is a delta function.

We can now compare the above derivation of the cylindrical diffusive motion to the steps of the more general derivations. Favro [1] indicated how to solve the rotational diffusion equation for the general anisotropic rigid body by the above Green's function method, which is straightforward as long as the diffusion operator, L , has a complete set of eigenfunctions, Ψ_n . Favro [1] noted that the anisotropic rotational diffusion operator is analogous to the Hamiltonian for a quantum mechanical anisotropic rigid rotor and he showed how to expand the eigenfunctions for the anisotropic rotor, Ψ_n , in terms of the eigenfunctions of a symmetric rotator, Φ_{nm} [37, 38]. He has tabulated the expansion coefficients and the corresponding eigenvalues in terms of the principal axes of the diagonalized diffusion tensor [1].

With these general comments in mind we now recapitulate the major steps in the derivation of the simple case and point out the analogous steps in the general derivations of ER and CE. The references to equations in this work will be in parenthesis.

1. The diffusion equation (Eq. (1)) appropriate for the geometry at hand is chosen. This is Eq. (1) of CE and Eq. (2.2) of ER.
2. The eigenfunctions and eigenvalues (Eq. (2)) of the diffusion operator, L , are found. These were found by Favro [1], and are defined by Eq. (5) of CE and Eq. (2.8) of ER. For the anisotropic case these functions must be expanded in terms of the symmetric eigenfunctions (see also Favro [1]).
3. The general solution is then expressed in terms of a sum of these eigenfunctions and the exponential time decay functions (Eq. (2)). Each eigenfunction has a particular time constant, or a set of, exponential time constants, associated with it, so that each term of the sum is a solution of the diffusion equation. These eigenfunctions represent angular distributions of the excited molecules, which will decay with one time constant. See Eqs. (3), (4), (9) and (10) of CE, and Eqs. (2.9) and (2.10) of ER (in Eq. (2.9) of

ER the integration should be over the Ω_0 variable rather than Ω .

4. The initial conditions are then considered (Eqs. (4), (5) and (2)), which determine the initial distribution of molecules. This is essentially a straight-forward geometric problem, and is done in CE by Eqs. (7) and (10), and in ER by Eqs. (3.2), (3.3) and (2.9).
5. The final signal is then calculated by projecting the time dependent solution for the distribution of emission dipoles onto the two axes of observation (Eq. (6)). Equations (8), (11) and (12) of CE, and Eqs. (3.2) and (3.4) (using Eqs. (3.5)–(3.8)) of ER. The axes onto which the emission dipoles are projected are the directions of polarization in the laboratory frame of reference that are used for measuring the anisotropy. These projections must be squared to represent the intensities of fluorescence.
6. The anisotropy is calculated (Eq. (7)), which is Eq. (13) of CE and Eq. (3.4) of ER.

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